

Differentiation of Single Aphid Cultured Sub-Isolates of Two South African *Citrus tristeza virus* Isolates from Grapefruit by Single-Strand Conformation Polymorphism

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ABSTRACT. In the Southern African Citrus Improvement Program, two mild *Citrus tristeza virus* (CTV) isolates (GFMS 12 and GFMS 35), derived from grapefruit, are used to pre-immunize all grapefruit selections to obtain cross-protection against severe CTV stem pitting strains which are readily transmitted by the endemic citrus vector, *Toxoptera citricida*. Nine GFMS 12 sub-isolates (12/1, 12/2, 12/3, 12/4, 12/5, 12/6, 12/7, 12/8, 12/9) obtained by single aphid transmissions have previously been shown to be significantly milder or more severe than the original isolate when biologically evaluated for growth and stem pitting in Mexican lime and Marsh grapefruit hosts. Fifty single aphid transmissions from GFMS 35 resulted in only two sub-isolates (35/1, 35/2), of which 35/2 produced significantly more stem pitting than the original population in Marsh grapefruit test plants. To identify genomic regions that differ in the sub-isolates when maintained in Mexican lime plants, single-strand conformation polymorphism (SSCP) analysis of genes p23, p25 and a small 281 bp fragment of p27 (p27B) was used. Only SSCP profiles of gene fragment p27B differentiated GFMS 12 and GFMS 35 sub-isolates, while the characteristic profiles of genes p23 and p25 for the original GFMS 12 and GFMS 35 isolates remained unchanged after single aphid transmissions. SSCP analysis of p27B differentiated GFMS 12 sub-isolates into two SSCP groups, while the SSCP profile for 35/2 differed from those for the original GFMS 35 isolate. Changes in the p27B SSCP profiles of single aphid transmitted sub-isolates consisted of the appearance of previously undetected DNA bands and/or disappearance of DNA bands present in the original isolates. The results suggest that single aphid transmissions have selected genomic RNA variants in the GFMS 12 and GFMS 35 populations, which gave rise to different populations that may differ in symptom expression. Comparison of SSCP profiles on the same gel using p25 and p27B revealed that the original isolates GFMS 12 and GFMS 35 can be differentiated from each other as well as from a mild (LMS 6) and a severe (GFSS 1) isolate maintained in Mexican lime plants.

Citrus tristeza virus (CTV), the most economically important viral pathogen of citrus worldwide, causes various disease syndromes of which stem pitting and decline are the most important (5). CTV virions are filamentous particles about 2000 × 11 nm in size, which are readily propagated through infected buds and transmitted by several species of aphids in a semi-persistent manner (4). Two capsid proteins of 25 and 27 kDa coat about 95 and 5% of the particle length, respectively (12). The CTV genome is a single-stranded, positive sense, RNA molecule with 19,226-19,296 nucleotides (nt) organized in 12 open reading frames (ORFs) encoding at least 17 proteins (17, 22, 35). CTV isolates are frequently mixed populations of different genotypes

(genomic RNA populations) from which variants with distinct properties can be selected (8, 23) and often contain multiple defective RNAs (dRNAs) that vary in size, abundance and sequence (2, 21, 37).

CTV is endemic in southern Africa due to the occurrence of the most efficient vector, *Toxoptera citricida* (Kirkaldy). In this citrus production region severe CTV stem pitting has a significant effect on yield and fruit size of grapefruit, resulting in the reduction of the economic viability of affected trees even when propagated on resistant rootstocks (10, 19, 20). Measures to reduce losses caused by severe CTV strains on the citrus cultivars in the Southern African Citrus Improvement Program (SACIP) include a quarantine system to avoid intro-

duction of exotic isolates and cross-protection with mild CTV isolates together with a certification scheme (36).

Two CTV isolates are currently used to pre-immunize grapefruit selections in South Africa, GFMS 12 and GFMS 35. Marais et al. (20) suggested that GFMS 12 contains several strains. The presence of a severe strain in GFMS 12 was revealed when single aphid transmitted sub-isolates of GFMS 12 were evaluated in Mexican lime and grapefruit hosts (34). Similarly, results from field experiments indicated that GFMS 35 might contain severe strains (32). A shift in strain dominance within a CTV population occurs apparently due to the influence of the host and/or environmental conditions, resulting in different effects on a host (10, 32). Therefore, severe strains that are present in a mild isolate used to pre-immunize grapefruit may become dominant with disastrous consequences. This has the potential to cause problems in southern Africa due to the diverse range of grapefruit selections used as well as the dramatic variation in climatic conditions between the different citrus producing areas of the region (6, 32). Therefore, continuous glasshouse and field evaluations of promising mild isolates for their protection abilities in different grapefruit selections as well as different climatic conditions are a necessary, but long term project (18, 24). The elimination of severe strains from GFMS 12 (and possibly GFMS 35) by the recombination of single aphid cultured sub-isolates that do not contain severe strains was proposed as an alternative strategy to obtain grapefruit cross-protection for the interim (34). Molecular profiles may be useful in addition to biological data when selecting sub-isolates that differ or which do not contain a severe strain.

The availability of the complete sequence of the genome of isolate T36 from Florida enabled research-

ers to detect genomic regions that differ between CTV isolates, using techniques based on reverse transcription-polymerase chain reaction (RT-PCR) and electrophoresis without sequencing (17). Restriction fragment length polymorphism (RFLP) analysis of cDNA of the coat protein genes was used to differentiate between CTV isolates differing in biological properties and geographical origin (16). Single-strand conformation polymorphism (SSCP) analysis allows the rapid differentiation of DNA fragments of the same size but with point mutations (26). This technique allowed detection of differences in the coat protein gene (29) as well as in several other genes of different CTV isolates (3, 9, 14, 30, 31). SSCP was demonstrated to be a useful tool for the analysis of the CTV genomic RNA populations in different parts of infected plants, in different hosts and before and after single aphid transmissions (3, 9, 34).

In this work, different CTV genes were analyzed using the SSCP technique to compare the genomic RNA populations of the two approved South African grapefruit cross-protecting isolates, GFMS 12 and GFMS 35, before and after single aphid transmissions. The correlation of SSCP profiles of the different sub-isolates with variations in stem pitting development in CTV sensitive indexing hosts was also investigated.

MATERIALS AND METHODS

CTV sources. Main isolates: The two grapefruit pre-immunizing isolates approved for the SACIP were used in this study. GFMS 12, derived from Nartia (Marsh selection) grapefruit, is used to pre-immunize all white and pigmented grapefruit selections while GFMS 35, derived from Redblush grapefruit, is used to pre-immunize all the red grapefruit selections. LMS 6, which was derived from Mexican lime and approved for lime, sweet orange and mandarin mild strain

cross-protection as well as a severe isolate derived from grapefruit, GFSS 1, were included for SSCP comparisons. CTV sources were established by bud-inoculating virus-free Mexican lime plants in an insect-free greenhouse at a temperature range of 24 to 28°C.

Sub-isolates: Nine GFMS 12 sub-isolates (12/1, 12/2, 12/3, 12/4, 12/5, 12/6, 12/7, 12/8, 12/9) obtained in a previous study (34) were used. The procedure used by van Vuuren et al. (34) was followed to obtain sub-isolates of GFMS 35 and for evaluation of their severity by biological indexing.

Isolation of double-stranded RNA (dsRNA) from infected tissue. Pooled samples of bark and midrib tissue (4 g) were frozen with liquid nitrogen and pulverized, and dsRNA was extracted by the phenol-detergent method of Dodds et al. (11) with minor modifications. The aqueous phase containing nucleic acids was adjusted to 16.5% ethanol, incubated at -20°C for 1 h and then centrifuged at 8000 g for 20 min to remove particulates. Nucleic acid preparations enriched in dsRNA were obtained by non-ionic cellulose column chromatography (CF-11; Whatman International, Maidstone, England). Cellulose and protein contamination was prevented by an additional phenol extraction step.

Finally dsRNA was concentrated by ethanol precipitation and resuspended in 50 µl sterile distilled water.

Reverse transcription-polymerase chain reactions (RT-PCR). For molecular characterization and differentiation of CTV isolates and sub-isolates, genes p23, p25, p27, and a small fragment of gene p27, p27B (13), were selected. Complementary DNA was synthesized from dsRNA by reverse transcription and PCR amplification using the Titan One Tube RT-PCR System (Roche Diagnostics, GmbH) and primers based on the sequence of Florida isolate T36 (Table 1) (27).

Two microliters of dsRNA was heat-denatured in the presence of 0.4 µM of each primer for 5 min, chilled on ice for 5 min and annealed at room temperature for 30 min. One-step RT-PCR was performed in a 25 µl reaction mixture containing 1 × RT-PCR reaction buffer (with 1.5 mM MgCl₂ and DMSO; Roche Diagnostics, GmbH), 0.2 mM of each dNTP, 5 mM DTT, and 0.5 µl enzyme mix (AMV reverse transcriptase and Expand High Fidelity enzyme mix; Roche Diagnostics, GmbH) in addition to the dsRNA-primer mix.

Thermocycling conditions were: 30 min at 50°C for RT (1 cycle), 3 min at 94°C (1 cycle), and 30 cycles of 30 s at 94°C, 20 s at 50°C and 60 s

TABLE 1
NUCLEOTIDE SEQUENCE OF PRIMERS USED FOR CDNA SYNTHESIS AND POLYMERASE CHAIN REACTION AMPLIFICATION

Primers ^z	Gene amplified	Primer sequence (5' → 3') ^y
CTV-CPL(+) CTV-CPR(-)	p25	ATGGACGACGAAACAAAG TCAACGTGTGTTGAATTT
CTV-p27L(+) CTV-p27R(-)	p27	ATGGCAGGTTATACAGTAC CTATAAGTACTTACCCAAATC
P27-E-3(+) ^x CTV-p27R(-)	p27B	CAAACGCTCTACGTAAGT CTATAAGTACTTACCCAAATC
CTV-p23L(+) CTV-p23R(-)	p23	TCTGCGAGTTACAATGG TCAGATGAAGTGGTGTTCGCGGAGAATCC

^zPrimers specific for each gene are listed in pairs.

^yPrimers specific for isolate T36 sequence from Florida (27).

^xPrimer selected by Gago-Zachert et al. (13).

at 68°C, to amplify p23, p25, p27 and p27B. In all cases, a final extension of 5 min at 68°C was used. RT-PCR products were visualized in 1% agarose gels stained with ethidium bromide.

SSCP analysis. For SSCP analysis, a modified procedure described by Yap and McGee (38) was followed. One microliter of the RT-PCR product was mixed with 9 µl dH₂O and 1 µl denaturing solution (500 mM NaOH, 10 mM EDTA pH 8.0). The mixture was heated for 10 min at 42°C and 1 µl loading dye added (0.5% xylene cyanol [w/v] and 0.5% bromophenol blue [w/v] in deionized formamide). Denatured DNA of the p23, p25 and p27 RT-PCR products were separated by electrophoresis in a non-denaturing 6% polyacrylamide minigel without glycerol, using 0.5 × TBE (44.5 mM Tris-Borate, 1 mM EDTA pH 8.0) as electrophoresis buffer and 200 V for 4 h at 8°C. The electrophoretic conditions for DNA strands of p27B RT-PCR products were 12% polyacrylamide without glycerol, 0.5 × TBE, 200 V, 4 h, 8°C. Gels were stained with silver nitrate (7).

RESULTS

GFMS 35 sub-isolates. Two sub-isolates (35/1, 35/2) were obtained out of 50 single aphid transfers. Their ELISA readings (titer) and biocharacterization in

two hosts are compared with the original isolate in Table 2.

Differentiation of GFMS 35 sub-isolates. Genes p23, p25, p27 and the small gene fragment, p27B, were compared by SSCP analysis. The RT-PCR products obtained using dsRNA and primers CTV-p23L/CTV-p23R (for p23), CTV-CPL/CTV-CPR (for p25), CTV-p27L/CTV-p27R (for p27) and p27-E-3/CTV-p27R (for p27B) (Table 1) were of the expected size.

SSCP analysis of the p25 gene in the original GFMS 35 isolate and sub-isolates 35/1 and 35/2 revealed that electrophoretic profiles did not change after single aphid transfers and consisted of only two intense DNA bands when maintained in the Mexican lime host (Fig. 2A, lanes 1-3). SSCP analysis of the p23 gene in the original GFMS 35 isolate and sub-isolates 35/1 and 35/2 also produced unchanged SSCP profiles. Each profile consisted of more than two DNA bands at varying intensities, suggesting that the genomic RNA population of GFMS 35 contained sequence variants at a detectable titer (Fig. 2A, lanes 7-9).

The p27 SSCP profile for sub-isolate 35/2 was altered from GFMS 35 after single aphid transmission (Fig. 2A, lane 6) while the p27 SSCP profile from sub-isolate 35/1 did not differ from that of the original GFMS 35 isolate (Fig. 2A, lane 5). Changes in the 35/2 profile consisted of the

TABLE 2
THE EFFECT OF GFMS 35 *CITRUS TRISTEZA VIRUS* ISOLATE AND TWO SUB-ISOLATES ON THE GROWTH AND STEM PITTING DEVELOPMENT OF MEXICAN LIME AND MARSH GRAPEFRUIT SEEDLINGS²

Isolate and sub-isolates	ELISA (OD ₄₀₅)		Growth (cm)		Pitting/cm ²	
	Mexican lime	Mexican lime	Marsh grapefruit	Mexican lime	Marsh grapefruit	
GFMS 35	1.589	33.8 ab	45.3 a	19.9 a	2.4 b	
GFMS 35/1	1.068	26.5 b	46.0 a	22.6 a	3.3 ab	
GFMS 35/2	1.066	37.0 a	37.5 a	21.3 a	14.2 a	

²Figures in each column followed by the same letter do not differ significantly at the 5% level (Fisher's LSD).

absence of bands present in the original GFMS 35 isolate (Fig. 2A, lane 4-6). To locate the variability detected in the p27 gene, a small 281 bp fragment at the 3' end of gene p27 (p27B) was analyzed by SSCP. Two DNA bands present in the original GFMS 35 isolate and sub-isolate 35/1 were absent in the single aphid transmitted sub-isolate 35/2, indicating that at least some of the variability detected in gene p27 is located in this fragment (Fig. 2B).

The complete p27 gene was also analyzed by RFLP analysis. Distinct DNA bands present in sub-isolate 35/1 and the original GFMS 35 isolate RFLP profiles differed for sub-isolate 35/2 after digestion with restriction enzyme *DdeI* (Roche Diagnostics, GmbH) (data not shown).

Differentiation of GFMS 12 sub-isolates. Gene p23 and a small fragment of gene p27 (p27B) were amplified by RT-PCR and analyzed by SSCP. No RT-PCR product was obtained when primers CTV-p27L and CTV-p27R (Table 1) were used.

SSCP analysis of gene p23 RT-PCR products revealed that the original GFMS 12 isolate and nine single aphid transmitted sub-isolates had an unchanged profile consisting of two intense DNA bands (Fig. 1A). Variability, however, was detected in gene fragment p27B when SSCP profiles from the original GFMS 12 isolate and single aphid transmitted sub-isolates were compared. Each profile consisted of two intense DNA bands (Fig. 1B), however, several light DNA bands varying in intensity and position appeared after longer silver stains (Fig. 1C). The p27B SSCP profiles from three sub-isolates 12/4, 12/5 and 12/8 showed similar changes consisting of the disappearance of the two intense DNA bands and the appearance of two new intense DNA bands at different positions when compared with the profile for the original GFMS 12 isolate (Fig. 1B, lanes 2-4). An additional intense DNA band appeared in the profile of

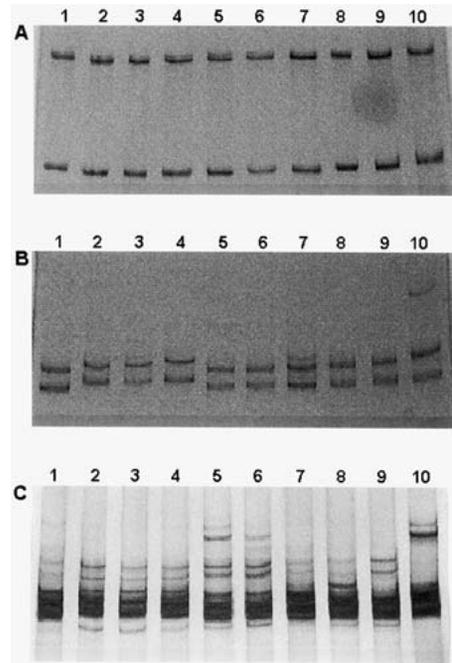


Fig. 1. Single-strand conformation polymorphism (SSCP) patterns of gene p23 (A) and fragment p27B of gene p27 (B and C) from original *Citrus tristeza virus* (CTV) isolate GFMS 12 and nine sub-isolates (12/1-12/9) obtained by single aphid transmissions. Complementary DNA was obtained by RT-PCR using primers CTV-p23L/R (for p23) and p27-E-3/CTV-p27R (for p27B) and dsRNA from a pool of infected tissue. Tracks in A, B and C are as follows: (1) original GFMS 12; (2) 12/4; (3) 12/5; (4) 12/8; (5) 12/2; (6) 12/6; (7) 12/1; (8) 12/3; (9) 12/7; (10) 12/9. Denatured DNA fragments were separated in a polyacrylamide gel (6 or 12% acrylamide) and stained with silver nitrate until intense DNA bands appear (A and B) or stained for longer periods (C).

sub-isolate 12/9 (Fig. 1B, lane 10) and was also visible as light bands at the same position in the profiles of sub-isolates 12/2 and 12/6 but not in the original GFMS 12 isolate when the gel was stained for longer periods (Fig. 1C, lanes 1, 5 and 6).

Comparison of the SSCP profiles of p25 and p27B in the original isolates. SSCP profiles of gene p25 and gene fragment p27B for the original mild isolates GFMS 12 and GFMS 35 were compared on the same gel. Profiles were also com-

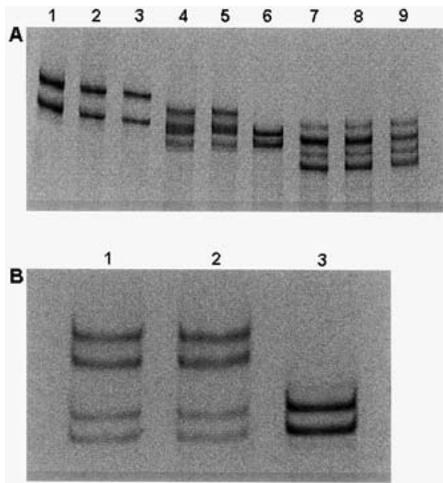


Fig. 2. Single-strand conformation polymorphism (SSCP) patterns of genes p25, p27, p23 (A) and fragment p27B of gene p27 (B) from original *Citrus tristeza virus* (CTV) isolate GFMS 35 and two sub-isolates (35/1 and 35/2) obtained by single aphid transmissions. Complementary DNA was obtained by RT-PCR using dsRNA as template and primers CTV-CPL/R (for p25), CTV-p27L/R (for p27), CTV-p23L/R (for p23) and p27-E-3/CTV-p27R (for p27B). Tracks in (A) are as follows: (1) original GFMS 35, p25; (2) 35/1, p25; (3) 35/2, p25; (4) original GFMS 35, p27; (5) 35/1, p27; (6) 35/2, p27; (7) original GFMS 35, p23; (8) 35/1, p23; (9) 35/2, p23. Tracks in (B) are as follows: (1) original GFMS 35, p27B; (2) 35/1, p27B; (3) 35/2, p27B. DNA fragments were separated in a polyacrylamide gel (6 or 12% acrylamide) and stained with silver nitrate.

pared with that of mild isolate LMS 6 used in a previous single aphid transmission study (34) and severe isolate GFSS 1.

Comparisons of p25 SSCP profiles revealed that both isolates GFMS 12 and GFMS 35 had a characteristic electrophoretic profile consisting of only two intense DNA bands, while profiles for the RNA genomic populations of LMS 6 and GFSS 1 showed variation within the coat protein gene (Fig. 3A). When p27B SSCP profiles for the same isolates were compared; it was shown that the genomic RNA populations of GFMS 12 and GFMS 35 had variation within the p27B gene fragment. The GFMS 12 SSCP profile

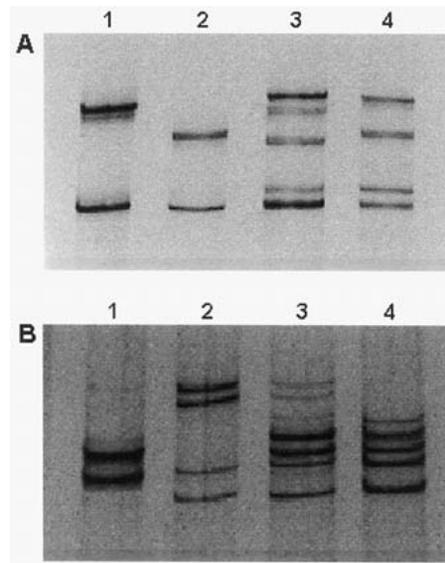


Fig. 3. SSCP patterns obtained of gene p25 (A) and fragment p27B of gene p27 (B) from original mild isolates GFMS 12, GFMS 35, LMS 6 and severe isolate GFSS 1. Complementary DNA was obtained by RT-PCR using dsRNA from a pool of infected tissue. Primers used were CTV-CPL/R (for p25) and p27-E-3/CTV-p27 (for p27B). Tracks in (A) and (B) are as follows: (1) GFMS 12; (2) GFMS 35; (3) LMS 6; (4) GFSS 1. Electrophoretic conditions were as in Fig. 2.

consisted of two intense DNA bands and several light DNA bands, while GFMS 35 had four characteristic intense DNA bands in its profile. The largest sequence variation in p27B as well as in p25 was observed for LMS 6, as deduced from the presence of multiple DNA bands in the profile (Fig. 3B). SSCP profiles of p27B and p25 for isolates GFMS 12, GFMS 35, LMS 6 and GFSS 1 are unique and can be used to differentiate among these isolates when maintained in Mexican lime plants.

DISCUSSION

Numerous strains of CTV exist and usually occur as a mixture within a host when grown in the field (25). Biological data of sub-isolates obtained by single aphid transmissions revealed that the two CTV

isolates approved for grapefruit pre-immunization in South Africa (34), GFMS 12 and GFMS 35, are composed of more than one strain. A GFMS 12 sub-isolate (12/3) was more virulent than the original isolate when indexed in Mexican lime and Marsh grapefruit hosts (34) and fell in the severe stem pitting category of a scale (more than 50 pits per cm²) used by Van Vuuren et al. (33). Also, significantly more stem pits developed in Marsh grapefruit test plants infected with sub-isolate 35/2 than with GFMS 35. However, sub-isolate 35/2 was classified as mild with the number of stem pits falling in the lower part (less than 20 stem pits per square cm) of the scale. Biological properties of a CTV isolate may depend on the effect of the prevailing strain in the viral population and if the strains in the population can be detected by molecular techniques, resulting profiles could be used to discriminate between sub-isolates obtained by single aphid transmissions. Aphid transmissions were reported to sometimes change the RFLP pattern of the coat protein gene (15, 16, 34), dsRNA pattern (1), SSCP profiles of randomly selected genes (3, 9, 34) and/or pathogenic characteristics (8) of CTV isolates. From these techniques, SSCP analysis was chosen for this study because of the ability to detect point mutations in similar genomic fragments (26). In contrast to the biological data, SSCP analysis of the p25 gene showed that the profiles of original isolates GFMS 12 and GFMS 35 remained unchanged in nine GFMS 12 sub-isolates (34) and two GFMS 35 sub-isolates obtained by single aphid transmissions (Fig. 2A). Furthermore, the SSCP profiles each consisted of only two characteristic bands, which suggested that their genomic RNA populations contained only one sequence variant at a detectable titer when maintained in Mexican lime.

In contrast, the SSCP profile of gene p25 from another mild South

African cross protecting isolate analyzed in a previous study, LMS 6, contained several DNA bands which changed in some single aphid cultured sub-isolates (34). SSCP analysis of gene p25 in a severe isolate from grapefruit, GFSS 1, also showed several DNA bands but the SSCP pattern was less complex than that of LMS 6 (Fig. 3A). It is therefore not possible as a rule to differentiate the three mild South African pre-immunizing isolates from the severe isolate based on their p25 SSCP profile complexity. On the contrary, Sambade et al. (31) observed that mild isolates included in their study tend to produce SSCP profiles with a single sequence variant using genes p18, p13, p20, p23, whereas severe isolates usually showed more complex patterns which suggest that the severe phenotypes are associated with higher genetic heterogeneity. However, SSCP profiles of gene p25 for isolates GFMS 12, GFMS 35, LMS 6 and GFSS 1 are unique and can be used to differentiate among these isolates when maintained in Mexican lime plants (Fig. 3A).

The p23 gene was also selected for SSCP analysis as this gene is suspected to play a regulatory role in the expression of other CTV genes and may serve as an indicator of disease severity (28). As was the case with p25, the SSCP pattern of p23 obtained from GFMS 12 consisted of only two DNA bands and remained unchanged after single aphid transfers. Conversely, GFMS 35 electrophoretic profiles for gene p23 contained at least four DNA bands at variable intensities but remained unchanged in the two sub-isolates. These results suggested that GFMS 12, like Spanish isolate T385 which contained highly predominant variants for genes p18 and p20, may have a viral population grouped around a single predominant genomic RNA variant (3, 9). D'Urso et al. (9) suggested that a viral population with such a struc-

ture may be less inclined to changes in graft and aphid transmissions.

The true structure of a viral population with a large genome can not be predicted from the molecular data obtained for only two genes known to be highly conserved. Genes responsible for CTV pathogenicity, plant movement and aphid transmissibility could be situated elsewhere on the CTV genome and show variability in their nucleotide sequence. A comparison between isolates T36 and VT genomic sequences indicated a gradual increase in sequence divergence toward the 5' terminus (22), therefore, the p27 gene was also selected for SSCP analysis. Gago-Zachert et al. (13) used gene p27 and two fragments of the gene, (p27A and p27B) for SSCP differentiation of six CTV isolates belonging to five different biogroups and to assess the genetic heterogeneity of each CTV population. Gene fragment p27B (3' terminal 281 bp of gene p27) showed a higher rate of sequence variation than the p27A fragment (5' terminal 459 bp of gene p27) and was also chosen for SSCP analysis in this study. The complete p27 gene from the GFMS 12 isolate could not be amplified using the conditions described in this study, while this gene was readily amplified for GFMS 35. Gene fragment p27B, however, was amplified for both GFMS 12 and GFMS 35. These results suggest differences in the p27 gene 5' terminal primer recognition site for GFMS 12 compared to those for GFMS 35 and Florida isolate T36.

For both isolates, GFMS 12 and GFMS 35, SSCP analysis of gene fragment p27B allowed differentiation of some sub-isolates from each other as well as from the original isolates. When comparing the two intense DNA bands, GFMS 12 sub-isolates could roughly be differentiated into two distinct groups, namely 12/4, 12/5, 12/8 in the first group and 12/1, 12/2, 12/3, 12/6, 12/7, 12/9 in the second group (Fig. 1B).

The sub-isolates within group one caused only mild stem pitting symptoms in Mexican lime and Marsh grapefruit test plants, while those in group two fell in the mild (less than 20 pits per square cm), intermediate (20-50 pits per square cm) and severe (more than 50 pits per square cm) stem pitting scale (33, 34). The light DNA bands appearing in the SSCP profiles after longer gel stains confirmed that 12/4, 12/5, 12/8 in group one are similar; while 12/2, 12/6 are similar and 12/1, 12/3, 12/7, 12/9 unique in group two (Fig. 1C). There was, however, no correlation between the unique profiles in group two and stem pitting severity.

Sub-isolate 35/2 could be differentiated from the original GFMS 35 isolate using both gene p27 and gene fragment p27B in SSCP analysis. The altered profile for 35/2 correlates with the significantly higher number of stem pits developing in Marsh grapefruit infected with 35/2 than with the original GFMS 35 isolate (Fig. 2; Table 2).

The absence of DNA bands in both the p27 and p27B SSCP profiles for sub-isolate 35/2 which is present in those for the original GFMS 35 isolate indicates that some of the sequence variability detected in the complete gene is situated in the 3' terminal 281 bp.

RFLP analysis of the complete p27 gene digested with restriction enzyme *DdeI* also differentiated 35/2 from the original GFMS 35 isolate with distinct DNA bands absent in the profile for sub-isolate 35/2 (data not shown). Some of the nucleotide changes detected for gene p27 in sub-isolate 35/2 by SSCP analysis therefore occurred within *DdeI* recognition sites.

SSCP analysis of p27B for GFMS 12, GFMS 35, LMS 6 and GFSS 1 compared on the same gel revealed that these isolates can be differentiated from each other. LMS 6, as was the case with p25 electrophoresis patterns, produced a more complex band pattern than severe isolate

GFSS 1 (Fig. 3B). Gene fragment p27B is more variable than the p25 gene and therefore useful to monitor the status of the GFMS 12 and GFMS 35 genomic RNA populations before and after single aphid transmission, graft transmission and challenges with severe CTV strains when maintained in Mexican lime plants.

The results indicate that GFMS 12 and GFMS 35 populations of genomic RNA variants after single aphid transmissions gave rise to different populations that may also differ in symptom expression. The gene(s) responsible for symptom

expression needs to be identified and characterized. Only then will it be possible to differentiate isolates and sub-isolates with different biological characteristics with rapid molecular techniques.

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